INTERACTIONS OF THE TAN SPOT FUNGUS, *PYRENOPHORA TRICHOSTOMA*, AND SELECTED MICROORGANISMS

By

JAMSHID GHAZANFARI-HESAMABADI

Bachelor of Science
Pahlavi University
Shiraz Iran
1967

Master of Science
Pahlavi University
Shiraz Iran
1972

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
DOCTOR OF PHILOSOPHY
July, 1983

INTERACTION OF THE TAN SPOT FUNGUS,

PYRENOPHORA TRICHOSTOMA, AND

SELECTED MICROORGANISMS

Thesis Approved:

Francis J. Gough
Thesis Adviser

Dallas E. Wadsworth

H. A. Melouk

Marion K. Hanchey

Norman N. Durham
Dean of the Graduate College

ACKNOWLEDGMENTS

The author wishes to express his appreciation to his major adviser, Dr. Francis J. Gough, for his guidance and assistance throughout this study. Appreciation is also expressed to Dr. Hassan A. Melouk, for his assistance and encouragement, and to Dr. Mark Sanborn and Dr. Dallas F. Wadsworth for their constructive criticism.

Gratitude is expressed to my wife, Mahin, our sons, Kambiz, Hooman and Babak for their understanding patience and sacrifices.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Yellow leaf spot, or tan spot, of wheat is a fungal disease that occurs on all wheat growing continents. In many parts of the world it is considered a problem in common and durum wheats of both spring and winter growth habit (15, 32, 33, 35, 45, 57). Bromegrass, wheat grass, wheat, rye, and barley are hosts of the causal fungus (57). Although tan spot can be serious alone, most frequently it is a component of a complex of leaf spotting diseases.

Symptoms of the disease appear on wheat leaves during the spring and early summer on small brown flecks which expand into dark-centered lenticular tan spots. The spots often coalesce and infected leaves, or portions of them, become necrotic and die.

The casual fungus, Pyrenophona trichostoma (Fr.) Fckl., [syn. P. tritici-repentis (Died.) Drechs.]; imperfect state, Drechslera tritici-repentis (Died.) Shoem., [syn. Helminthosporium tritici-repentis (Died.) and H. tritici-vulgaris Nis.] grows on various artificial media. It produces greyish-white mycelial colonies with numerous immature ascostromata in and on most media. Matured ascostromata containing asci and ascospores are produced within 25 days on sterile wheat straw incubated at 16 C under light. In nature, P. trichostoma grows saprophytically on host debris. Ascospores, conidia and hyphal fragments may serve as primary inoculum, and leaf, infections are numerous when host plants

are grown in close proximity to host plant residues (57).

Since 1971, when tan spot was first considered as economically important in North Dakota, it has been reported as a serious foliar disease of wheat on the Central Plains of North America (21, 24, 44, 53, 56,). There are at least three reasons why this disease became recognized as important in recent years. Firstly, leaf symptoms usually occur interspersed among those of other diseases, and because they bear a gross resemblance to certain developmental phases of Septoria induced lesions they were overlooked. Secondly, in recent years new farming practices which leave crop residue on the soil surface have enhanced the amounts of primary inoculum. Thirdly, and perhaps most importantly, new wheat genotypes with increased susceptibility have been grown on large land areas.

The sexual stage and a large portion of the annual vegetative life of P. trichostoma exists saprophytically in host residue. In Oklahoma, this saprophytic state exists from mid-June of one year to mid-March and beyond of the following year. During this time, its residential host debris is exposed to cellulose and lignin degradation enzymes produced by microorganisms. It is logical to assume that among the various microorganisms which degrade the residue (and those that utilize degradation products) there are those which are antagonistic to growth and reproduction of P. trichostoma. Skidmore (47) states that,

> The start of any investigation into micro-biological control of pathogens is to search for potential antagonist in the habitat in which the pathogen is normally found. This search involves an assessment of the population in the habitat, both in terms of species composition and numbers of propagules (p. 507).

Work described in this dissertation is concerned with; a) the

isolation from soil and plant parts of fungi and bacteria antagonistic to growth and reproduction of P. trichostoma, b) their identification, and c) their relative antagonistic potency.

CHAPTER II

LITERATURE REVIEW

Wheat occupies approximately 20% of the world's cultivated land, and provides about 20% of the world's food calories. It is the staple food for nearly 40% of the worlds population.

The actual number of wheat diseases is unknown, but about 50 are commonly considered as economically important. Tan spot is among these (57).

The causal fungus of tan spot, P. trichostoma, belongs to the class Ascomycetes. During the past few decades concepts regarding relationships within the ascomycetes has undergone several revisions. Recent studies have focused on details of ascocarp formation. Ascomycetous fungi belonging to the class Loculoascomyctes have been arranged into eight orders by Barr (3). She placed the genus Pyrenophora in the Loculoascomyctes as follows: family, Pyrenophoraceae; suborder, Pleosporineal; order, Pleosporales; and subclass, Loculoascomycetidae. Wehmeyer (56) describes P. trichostoma as follows:

> Pyremophora trichostoma is a common species on grasses. The spores are variable in size. The setae are more common on young or sterile stromata and are reported to bear conidia, which have been shown to belong in the genus Helminthosporium. These setae are brittle and break off easily; they may be entirely lacking on some stromata. This species has been studied extensively by pathologists of its pathogenicity on many grasses, particularly the cereal grains.
>
> Ascostromata 300-500 (750) x 200-350 μ, flattened-globose, sclerotoid, usually rather thickly scattered, strongly erumpent, often becoming superficial, wall thickened

> (50-100μ), especially at the sides; ostiole punctate, upper surface often with short or longer, stiff, brown, pointed, septate setae, which are said to bear conidia, but break off easily and are sometimes wanting.
>
> Asci 160-210 x 35-50μ, stout-clavate, becoming elongate with a much thickened wall, and clavate base, with some interthecial tissue. Spores 35-60 x 15-25μ, biseriate, fusoid to oblong, sometimes clavate-ellipsoid, light yellow-brown, ends broadly rounded or abruptly tapered, 3-septate slightly constricted at the septa, with a vertical wall in one or both central cells but not in the end cells (pp. 280-281).

A yellow spot disease of winter wheat coused by H. tritici-vulgaris was reported to have occurred in Michigan in 1951 (1). No sexual stage was reported.

Hosford's (21) report of P. trichostoma in North Dakota appears to be the first to attribute severe wheat yield losses to this fungus in the United States. Using Wehmeyer's (56) description of P. trichostoma, he concluded that pseudothecia formed on wheat stubble in the field were similar to those produced by a fungus he had isolated from tan leaf lesions. Subcultures grown on autoclaved wheat seed at 16 C for 47 days produced both immature ascostromata and mature ascostromata containing asci and ascospores.

Hosford (22) later stated that conidia and conidiophore characteristics of P. trichostoma fitted descriptions of H. tritici-repentis and H. tritici-vulgaris. Also, the similarity in culture and pathogenicity of isolates from these conidia and hyphae with isolates from ascospores, and from leaf spots, caused him to conclude that P. trichostoma, H. tritici-vulgaris and H. tritici-repentis are the same fungus. In addition, he noted (26) that P. trichostoma and Septoria avenae f. sp. triticea cause similar leaf spots which previously had been attributed only to Septoria in North Dakota. He stated further that, "The findings. . . would lead one to suspect that P. trichostoma was involved in the severe

leaf spotting attributed to Septoria in North Africa."

Thus, tan spot appears to be a world-wide pest of wheat. The fungus, often designated as H. tritici-repentis has been associated with damage to wheat in Canada (10, 11, 19), Japan (37), Israel (29), Cyprus (39), Kenya (15), Tanganyika (54), and India (35).

Leaf spotting caused by P. trichostoma caused measurable losses in common and durum spring wheats in North Dakota (24). Fungicide applications reduced severe leaf spotting by an average of 1.0 rating unit and increased yields by an average of 11.2%. Severe leaf spotting and yield losses are related to weather conditions which maintains moistens on the foliage for 30-48 hr. or more. Depending upon weather conditions the disease may cause major losses in both susceptible and tolerant or moderately resistant wheats (23, 24).

The severe damage and wide distribution of tan spot in Nebraska has been attributed to several factors (55). Among these factors are; a) susceptibility of the predominant wheat genotypes, b) monoculture, c) leaving wheat residue on the soil surface to reduce erosion and conserve water, and d) several periods of cool, wet, cloudy weather in the early growing season for sporulation and infection.

Platt et al (40) studied conidiation of P. trichostoma. Conidia developed with seven isolates when either 15% V-8 juice agar or autoclaved wheat leaves were the substrates. Conidiophores developed over a temperature range of 10 to 31 C, and conidia developed over a range of 10 to 25 C with an optimum of 21 C on V-8 agar. Conidiophores developed in any of 25 photo period regimes containing a light period. Conidia formed in 1 to 21 hr of light (4790 1x) per day with an optimum of 12$\pm$ 1 hr at 21 C on V-8 agar.

Mature pseudothecia developed on wheat straw after 2.5 to 3 wk under fluorescent light at 15-18 C (38). Pseudothicia only rarely produced asci after 4-6 wk in darkness, and failed to produce asci in either light or darkness at 24-26 C.

Reports of biological control of pathogens of aerial surfaces of plants are rare compared with those concerning subterranean plant parts (2). The paucity of reports on biological control of foliar pathogens may partially derive from an emphasis placed on fungicides to control foliar diseases. Currently, however, there is an increasing awareness of negative side-effects of chemicals on the ecosystem and a growing interest in pestiside-free agricultural products. These factors may spur development of biological control (17).

Investigation into micro-biological control of pathogens usually begins by a search for potential antagonists in the habitat in which the pathogen is usually found. A number of antagonistic saprophytes have been reported as occurring on foliar parts of different plants (47). Bacteria on plant surfaces may be either saprophytic or pathogenic.

Among filamentuous fungi, Alternaria spp. have been isolated from the phylloplane of many plants including wheat (17). Alternaris tenuis (A. alternata) has been reported as a phylloplane fungus (4, 9, 13). Direct examination techniques have shown that filamentuous fungi can grow on the surfaces of green leaves (12, 14). To relate the physiology of filamentous fungi commonly found on green leaves with their observed activity on leaf surfaces in vivo, Dickson and Bottomly (13) studied A. alternata and two other fungi isolated from wheat. They showed that A. alternata, in the absence of exogenous organic nutrients, germinates

rapidly in both free water and in very high humidities. Several fungi, including A. tenuis, have been evaluated for their ability to inhibit P. graminis tritici urediospores (34).

Skidmore and Dickson (48) examined interactions, on culture media, of fungi commonly found on barley foliage and other fungi previously studied for interactions. Interactions were measured against a scale based on observations of Porter (40), but modified to award values to fungi depending upon their antagonism. The two extreme ends of the scale were; a) when fungi grew into one another with no microscopic signs of interaction, and b) where fungi were mutually inhibitory at a distance of 2 mm or more. Each fungus was assessed for its "active" ability to inhibit the growth of another, and for its own response to antagonism, i.e., its status as a "passive" species. Assessments were made when the fungi had reached a state of equilibrium in which no further changes in growth patterns occurred.

In vitro inhibitory effects have been attributed to many causes including antibiotic production, pH changes, nutrient alterations, mechanical obstructions, and hyperparasitic interactions (47).

Numerous fungi produce either volatile or non-volatile antibiotics (18, 20, 25). These include Alternaria (31) and Helminthosporium (8).

There have been many attempts to control fungal pathogens with antagonistic bacteria, yeasts, or fungi. The mechanisms behind such antagonistic interactions vary, and many possibly overlap. For example, pH effects or antibiotic activity may be enhanced by nutrient enrichment of the substrate or may occur when plant tissues senesce.

CHAPTER III

MATERIALS AND METHODS

A V-8 juice agar medium was used in almost all experiments. The medium was prepared by adding 200 ml of V-8 juice and 3g of $CaCO_3$ to 800 ml of distilled water in a 1,000 ml graduated cylinder. The contents were shaken vigorously until the $CaCO_3$ and particulate matter in the V-8 juice were suspended evenly throughout the liquid. The suspension was then set aside for 3 hours, after which most solid material had settled to the bottom of the cylinder. The supernatant was decanted into another cylinder and its volume brought to 1,000 ml by adding distilled water. The liquid was then poured into a flask, 20g of agar added, and the whole autoclaved for 20 min at 121 C under 15 psi.

Single ascus and single ascospore-cultures of P. trichostoma were obtained from pieces of field infested wheat straw collected in March and kept on moist filter paper in petri dishes for about 2 wk at room temperature. Mature pesudothecia were dislodged from the straw and crushed on water agar with a flattened transfer needle. With the aid of a dissecting microscope, single asci and ascospores were transferred with a sharp needle from the water agar to V-8 juice agar.

A single ascus-culture, designated PYOK-14, was selected for use in this study. Unless otherwise specified, inoculum of PYOK-14 consisted of a 5 mm diameter plug of agar and mycelium taken approximately 2 cm from the edge of cultures on V-8 juice agar in petri dishes.

Microorganisms from soil, wheat straw, and living wheat leaves were assessed for antagonistic activity against P. trichostoma.

a) Isolation of antagonists from soil. Soil tested for the presence of antagonists were collected from fields and plots managed under different soil tillage and cropping systems. Samples were collected from the top 14 cm of soil. The procedure of testing for the presence of antagonists as follows: Fifteen g of each soil sample were placed in 150 ml of sterile distilled water in an Erlenmeyer flask (250 ml) and shaken for 30 min at 150 strokes per min with a reciprocal shaker. From each soil suspension, 1 ml was removed and added to a ml of sterile distilled water. From these initial dilutions, further dilutions were made serially to produce final dilutions of soil suspensions of 1:100,000 and 1:1,000,000. One ml of final dilutions of each soil sample was transferred to each of four sterile plastic petri dishes. Approximately 20 ml of pre-prepared V-8 juice agar, maintained at 45-47 C, was then poured into each petri dish and mixed with the soil suspension by gently shaking and swirling before the agar solidified. Inoculum of PYOK-14 was placed at the center of each petri dish. After 2 days at room temperature (20-24 C), growth of bacterial and fungal colonies, as well as PYOK-14, was evident in and on the medium. The plates were examined daily, organisms which appeared to inhibit growth of PYOK-14 were transferred to fresh medium at standard distances from inoculum of PYOK-14. Organisms which showed growth inhibitory properties were maintained for additional tests.

b) Isolation of antagonists from wheat straw. Wheat straw collected from wheat fields in the fall was incubated at 16 C (optimum temperature for pseudothecia production by P. trichostoma) on moist filter paper in petri dishes. As growth and sporulation of bacteria and fungi appeared

they were transferred to V-8 juice agar, and purified by repeated selection of cells, spores, or hyphal tips. Isolated cultures were passed through preliminary screening tests for inhibitory activity as described for microorganisms isolated from soil.

c) Isolation of potential antagonists from wheat leaves. Living leaves were randomly collected from field grown winter wheat in the tillering stage during March. The leaves were washed for 30 min in 150 ml of distilled water in an Enlenmeyer flask on a shaker set at 150 strokes per min. Ten ml of the wash water was transferred with a sterile pipette to each of 20 sterile petri dishes. Twenty ml of pre-prepared V-8 agar (45-47 C) was added to each dish and swirled gently. When the agar solidified, it was inoculated with PYOK-14 and incubated at room temperature. The cultures were examined as necessary for indications of inhibitory interactions between bacterial and fungal colonies with PYOK-14.

d) Evaluation of selected microorganisms for their ability to inhibit growth of P. trichostoma isolate PYOK-14. One bacterium and three fungi, because of their inhibitory effects on growth of PYOK-14, and because they were frequently associated with and isolated from soil samples and plant parts, were selected and tested for their relative ability to inhibit growth of PYOK-14. The inhibitors were identified as Bacillus lichemiformis (isolated from soil and wheat leaves), Bepolaris sorokiniana (isolated from soil and wheat straw), Stachybotrys chartarum (isolated from wheat straw) and Alternaria tenuis (isolated from wheat straw).

To facilitate measuring effectiveness of inhibitors a circular template 9 cm in diameter was drawn on white paper (Fig. 1). A point inside and 4 cm from the circle was marked as the position for inoculum of PYOK-14; opposite this point, and 3 cm from it, a tangential line 3 cm

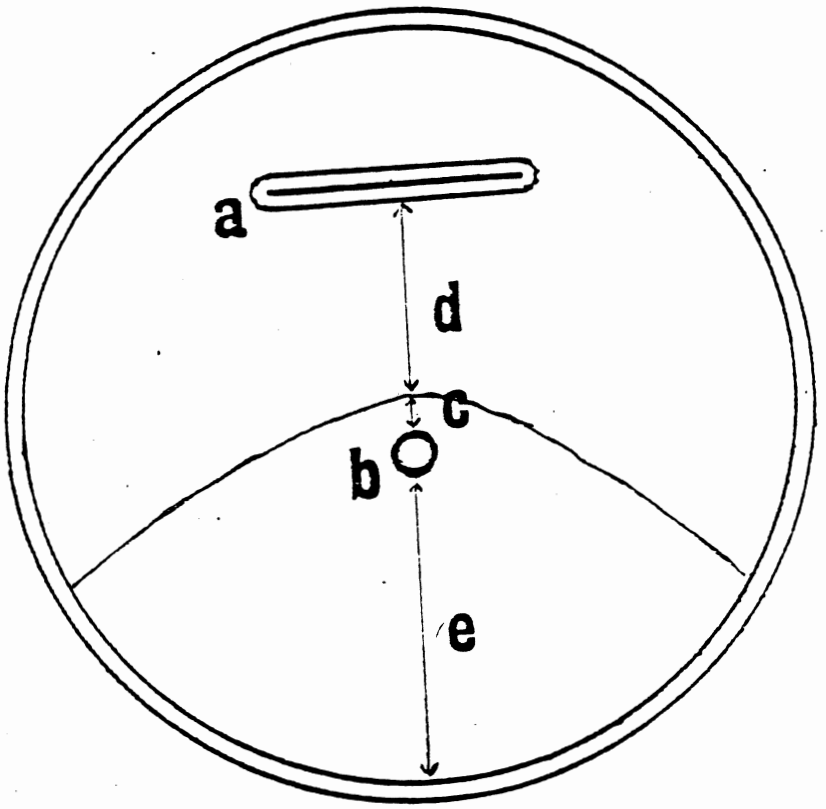

Figure 1. Template used to standardize placement of inoculum of antagonists (a) and Pyrenophora trichostoma (b) measurements (c), (d), and (e) were used to calculate growth inhibition and antimicrobial substance indices.

long was drawn to indicate the site for inoculum of each inhibitor. By placing each petri dish over the template the inhibitors could be placed precisely in each dish.

Medium in four petri dishes were inoculated with each combination of isolate PYOK-14 and inhibitor. One petri dish containing each combination (treatment) was assigned randomly to each of four replications. After one week of incubation at room temperature, three measurements were taken; namely, radial growth of PYOK-14 toward the inhibitor, radial growth of PYOK-14 toward the petri dish wall directly opposite the inhibitor, and width of the inhibition zone between the paired organisms. A growth inhibition index (GII) for each treatment was derived by dividing mm of growth of PYOK-14 toward the inhibitor (C) by mm of growth in the opposite direction (Fig. 1). Also, production of an antimicrobial substance (s) was presumed to cause the growth inhibition zones. Because B. licheniformis differed from the fungi in mode of growth, and the fungi grew at different rates, the width of the zones, per se, did not necessarily indicate relative potencies of the inhibitory substances. Thus, an antimicrobial substance index (ASI) was calculated by dividing the width of the zone (d) by radial growth of PYOK-14 on the side opposite of the inhibitor.

e) Evaluation of selected microorganisms as inhibitors of asocarp production by P. trichostoma. Ascocarp production by P. trichostoma on different media reportedly is best under moist conditions and continuous light at 16 C (38). In this study, these conditions were maintained by incubating cultures in a cold temperature incubator equipped with a fluorescent lamp, and by adding sterile distilled water to the media as needed.

Water agar, sterile wheat straw (autoclaved or treated with propylene oxide), sterile wheat straw on water agar, and sterile wheat straw on filter paper were tested on media for ascocarp production in petri dishes. On water agar alone, ascocarps developed and matured in about 40 days, but their numbers were fewer than those that developed on the other media. Ascocarps formed within 10 days and matured within 20-25 days on autoclaved straw or either water agar or wet filter paper. Sterile straw on moist filter paper was the medium of choice because of ease of preparation.

Straws of approximately equal diameter were cut into 2 cm lengths and 16 pieces were placed in each petri dish. Different arrangements of straw on the filter paper were tested. These included dipping the straws in cell or spore suspensions of the inhibitors and spreading them randomly, or in a radiating configuration, around inoculum of PYOK-14 at the center of the filter paper; grinding the straws into small particles and spreading them over a 2 cm x 4 cm area; and placing the straws side by side and in contact with each other (Fig. 2). These last two patterns provided both PYOK-14 and the inhibitors equal opportunity to grow and compete for nutrients. Preliminary tests indicated that placing the straws side by side was the most efficient arrangement both in terms of labor and consistency of results.

The straw medium was inoculated by placing inoculum (3 mm diameter plugs cut from petri dish cultures) of PYOK-14 and each inhibitor in opposition at the centers along the cut edges of the straws (Fig. 2). Each paired combination of PYOK-14 and an inhibitor, and each check was considered a treatment. One petri dish of each treatment was assigned

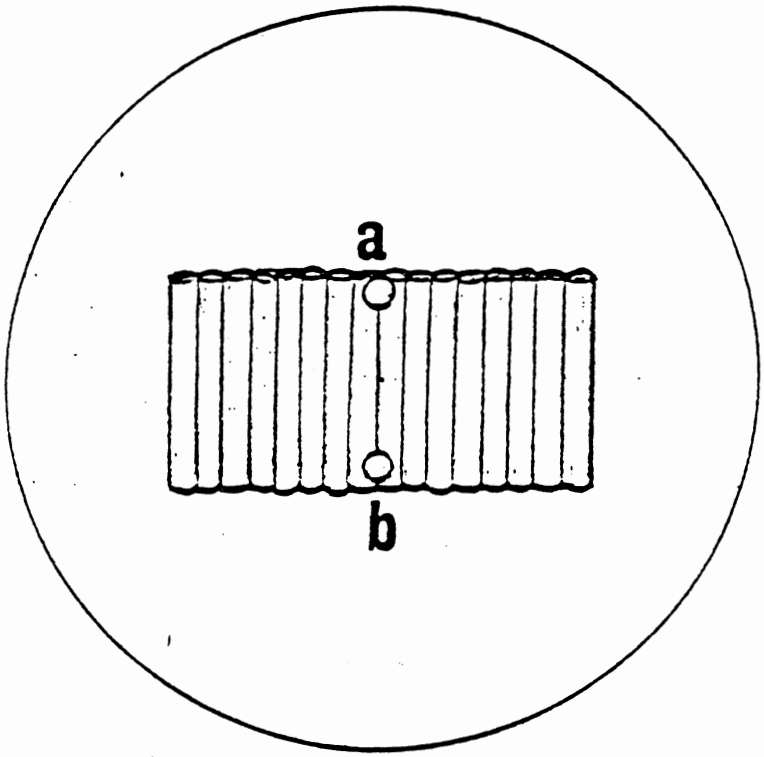

Figure 2. Arrangement of wheat straw segments in a petri dish for determining the effect of microbial antagonists (a) on pseudothecial production by Pyrenophora trichostoma (b).

to each of four replication. The cultures were incubated for 36 days at 16 C under constant light.

Thirty-six days after inoculation radial growth of PYOK-14 was measured along an imaginary line directly between the points of inoculation of PYOK-14 and the inhibitor, and in both directions at right angles to the line. The mean of these measurements were used as an estimate for growth of PYOK-14. Effects of inhibitors on ascocarp production was determined by counting all ascocarps visible on the straws without removing the straws from their positions on the filter paper.

f) Interactions among organisms inhibitory to growth and reproduction of P. trichostoma. The bacterium B. licheniformis, the most potent inhibitor of growth and reproduction of PYOK-14, was used to challenge growth of the three fungal inhibitors. Methods involved in the test were similar to those described for evaluating antagonistic effects of the inhibitors against PYOK-14.

g) The effect of B. licheniformis on infection of wheat seedlings inoculated with P. trichostoma isolate PYOK-14. Twelve pots of wheat seedlings of a tan spot susceptible cultivar, TAM W-101, were grown in a greenhouse. When the seedlings were 10 days old they were divided into three sets of four pots each. One set was inoculated with spores and fragmented mycelim of PYOK-14, a second set, similarly inoculated was immediately sprayed with a week-old culture of B. licheniformis in liquid V-8 juice medium, and the third set was sprayed only with the B. licheniformis culture. Inoculum of PYOK-14 was prepared by fragmenting mycelium grown in liquid V-8 juice medium, and applied to the plants as a spray applied with an atomizer. All plants were placed in a humidity chamber maintained at about 20 C and kept moist for 48 hrs. The

plants were then moved to a greenhouse for 7 days when effectiveness of B. licheniformis as a protectant against infection by PYOK-14 was evaluated.

h) Quantification of the inhibitory activity of B. licheniformis against P. trichostoma (on a cell basis). Bacillus licheniformis was grown in Difco Czapek dox broth on a reciprocatory shaker for 7 days. From the cultures, 0.1 ml of the cell suspension was added to 9.9 ml of sterile distilled water. From the latter, 0.1 ml was removed and added to another 9.9 ml, and the process repeated until a dilution series of 10^{-6}, 10^{-8}, and 10^{-10} was obtained. From each dilution, 0.1 ml was taken and added to 20 ml of V-8 juice agar in petri plates. Bacterial colonies that developed on the plates were counted after 1 wk. At the time the dilution series was prepared, the original bacterial suspension culture was placed in centrifuge tubes and centrifuged for 15 min at 10,000 rpm (16,300g) to pellet the bacterial cells.

The pelleted bacterial cells were discarded and the supernatant passed through a sterile millipore filter with a pore size of 0.45 µm. The filtrate was then added to V-8 juice agar in amounts to produce a series of 60, 40, 20, 10, and 1 percent concentrations of this filtrate. The agar plates were then inoculated with P. trichostoma. After one week of incubation, radial growth in all concentrations was too limited to obtain measurable growth differences. Consequently, a second experiment was performed using 1.0, 0.5, 0.25, and 0.125 percent of this filtrate in liquid V-8 medium. The medium was inoculated with P. trichostoma in agar plugs from a 1 wk old culture.

One week after the media were inoculated the mycelium of P. trichostoma was collected on filter paper, dried and weighed.

i) Partial purification and characterization of P. trichostoma growth inhibitor produced by B. licheniformis. Bacillus licheniformis was grown for 7 days in Czapek dox broth. The cultures were then centrifuged at 10,000 rpm (16,300g) for 15 min. The pellet of mostly bacterial cells was discarded. Ammonium sulfate crystals were added to the supernatant (60g/100 ml). After standing overnight the precipatate was collected by centrifuging the suspension at 17,500 rpm (37,000g). The pellet was dissolved in 0.01 M phosphate buffer (pH 6.0) and then dialyzed (cellulose dialysis tubing with mw cut-off 12000-14000 was used) against the same phosphate buffer, with several changes for 48 hrs in a refrigerator at 4°C. The mixture in the dialysis tubing was loaded on a sephadex gel (G-200) filtration column (42 cm length and 1.4 cm diameter). Blue Dextran 2000 was loaded before the sample to indicate the front of the eluting buffer. Fractions were collected every 30 min (5.6 ml). Fractions (100 µl) from columns (or from other stages of the purification process) were placed into 5 mm diameter holes cut in the center of 20 percent water agar in petri plates. After 24 hrs, the agar was sprayed with finely chopped mycelium of P. trichostoma. Forty-eight hrs later the plates were observed for evidence of inhibition zones

CHAPTER IV

RESULTS

(a) Isolation from soil of microorganisms antagonistic to growth of P. trichostoma. The most antagonistic organism observed, as indicated by growth inhibition zones (Fig. 3), was a light tan bacterium. When the bacterium occurred at 3 or more separated sites in the plates, inhibitory activity of other microorganisms was partially or totally masked. In some cases, the bacterium completely inhibited growth of P. trichostoma, isolate PYOK-14, beyond the inoculum plug. When isolated, the bacterium inhibited growth of PYOK-14 at a distance of about 20 mm. The bacterium was identified as Bacillus licheniformis, based on criteria in the 8th edition of Bergy's Manual of Determinative Bacteriology (19). The identity of B. licheniformis was confirmed by Janet S. Weaver, Department of Microbiology, Oklahoma State University.

Fungi also inhibited growth of PYOK-14 in the dilution plates. Unidentified species of Alternaria and Helminthosporium appeared to be the most inhibitory, although this may have been an artifact conditioned by a more rapid growth than that of other inhibitory fungi.

(b) Isolation of microorganisms antagonistic to P. trichostoma from wheat straw. Three fungi consistently associated with wheat straw were isolated and identified as Bipolaris sorokiniana, Stachybotrys chartarum, and Alternaria tenuis. Taxonomic keys by Shoemaker (46),

Figure 3. Inhibition of Pyrenophora trichostoma by Bacillus licheniformis as it appeared initially in a soil dilution plate.

Jong and Davis (28), and Joly (27) were used to determine the species of Bipolaris, Stachybotrys, and Alternaria, respectively.

(c) Isolation of microorganisms from living wheat leaves. Microorganisms isolated from living leaves of wheat plants included the yeasts, Cryptococcus sp. and Sporobolomyces roseus; the fungus Cladosporium sp. and an unidentified yellow bacterium. These microorganisms are common saprophytes on the surfaces of wheat leaves. They showed no inhibitory effects on growth of PYOK-14 when paired with that isolate on V-8 juice agar.

In addition to the above, B. licheniformis was isolated once from wheat leaves. It is probable that it originated at the soil surface and was transferred to the leaves by wind or splashing rain.

(d) Evaluation of selected microorganisms for their ability to inhibit growth of P. trichostoma on V-8 agar medium. Growth of PYOK-14, based on the growth inhibition index (GII), was inhibited equally well by B. sorokiniana and B. licheniformis. Similarly, S. chartarum and A. tenuis were equally proficient at inhibiting growth. Bipolaris sorokiniana and B. licheniformis were significantly superior to both S. chartarum and A. tenuis (Table 1). Bipolaris licheniformis stopped radial growth of PYOK-14 from a distance of 12-20 mm with never an intermingling of colonies (Fig. 4 and 5). Likewise, B. sorokiniana and S. chartarum stopped growth of PYOK-14 at a distance without mycelial contact (Figs. 6 and 7). In paired cultures of PYOK-14 and A. tenuis, hyphae of the two organisms intermingled, indicating that reduced growth of PYOK-14 probably derived from direct competition for nutrients, rather than production of antibiotics or toxic substances.

Variation in method and rates of growth among inhibitors precluded

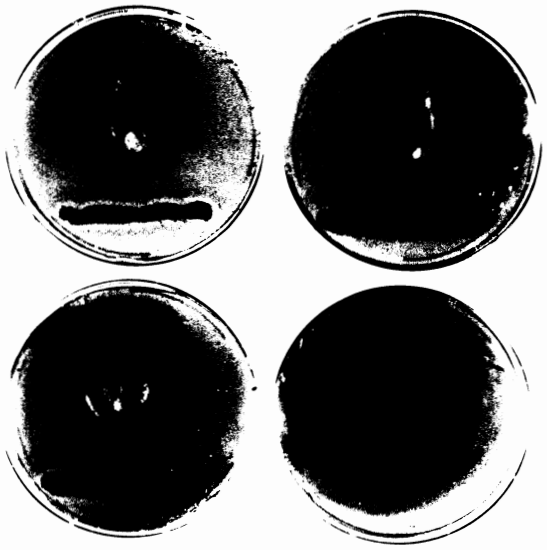

Figure 4. Inhibition of growth of Pyrenophora trichostoma by Bacillus licheniformis from a distance without intermingling of colonies. Petri plate at lower right contains Pyrenophora trichostoma grown singly.

Figure 5. Inhibition of growth of Pyrenophora trichostoma by Bipolaris Sorokiniana without mycelial contact.

Figure 6. Inhibition of growth of Pyrenophora trichostoma by Stachybotrys chartarum without mycelial intermingling.

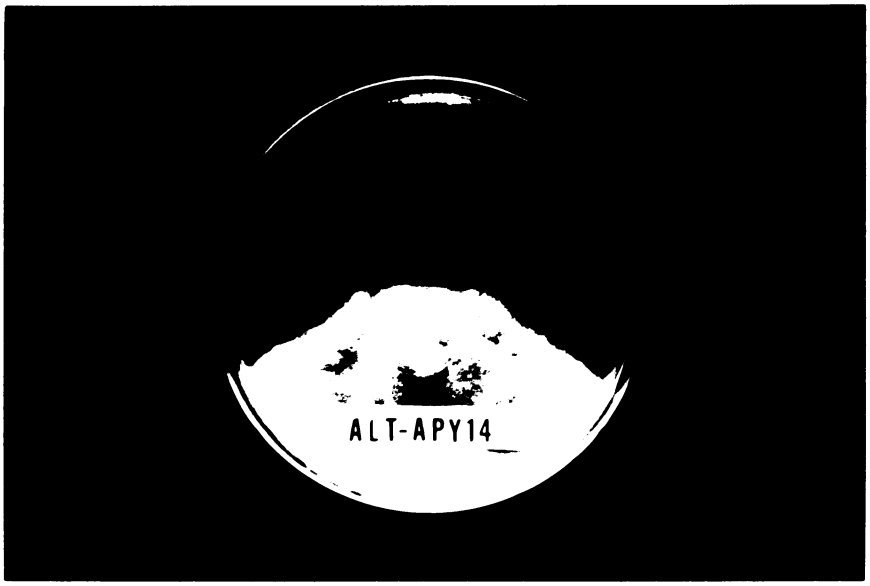

Figure 7. Inhibition of growth of Pyrenophora trichostoma by Alternaria tenuis with an intermingling of mycelium.

radial growth and widths of inhibition zones, per se, to accurately convey relative potency of growth inhibitory substances. Thus, an antimicrobial substance index (ASI) was calculated (Table 1). Based on ASI values, the growth inhibiting substance released by B. licheniformis was significantly more effective than those produced by either B. sorokiniana or S. chartarum at the 0.05 confidence levels; while the inhibitory product of B. sorokiniana was more effective than that of S. chartarum at the 0.05 level.

(e) Evaluation of selected microorganisms for their ability to reduce pseudothecia production by P. trichostoma - Experiment 1. The average numbers of pseudothecia (four replications) produced by PYOK-14 on sterile wheat straw, nonsterile straw from the field, and on sterile straw inoculated additionally with each growth inhibition microorganism fitted into three nearly discreet groups at 0.05 confidence level, according to the Newman-Kuel procedure (Table 2). The fewest pseudothecia were produced on nonsterile straw and on sterile straw inoculated with B. sorokiniana, B. licheniformis and A. tenuis. The mean numbers of pseudothecia per cm^2 (14.1 $\pm$ 1.9) produced within this group of culture combinations was about 28% of the number of pseudothecia produced on sterile straw alone. An intermediate number of pseudothecia, about 45% of the number on sterile straw, was produced in cultures containing S. chartarum and PYOK-14. S. chartarum grows very slowly in comparison to the other two fungi; consequently, PYOK-14 made more radial growth before bring halted by antagonistic action and thus occupied a large surface area in which to produce pseudothecia.

Experiment 2. In addition to differences in numbers of pseudothecia produced by PYOK-14 on sterile straw, growth inhibition zones were

Table 1. Radial growth of Pyrenophora trichostoma as influenced by four microorganisms and their production of antimicrobial growth substances in paired cultures on V-8 agar medium.[a]

Treatment (Inhibitory micro-organism)	Growth Inhibition Index[bde]	Antimicrobial Index[cde]
Bacillus licheniformis	0.36 t	0.79 x
Bipolaris sorokiniana	0.33 t	0.23 y
Stachybotrys chartarum	0.78 u	0.08 z
Alternaria tenuis	0.84 v	--
Pyrenophona trichostoma(ck)	1.00 w	--

[a]All measurements are means of four replications.

[b]Growth inhibition index (GII) = radial growth of P. trichostoma toward inhibitor ÷ radial growth in opposite direction.

[c]Antimicrobial substance index (ASI) = width of inhibition zone ÷ radial growth of P. trichostoma opposite the inhibitory micro-organism.

[d]GII and ASI values were not calculated from measurements made in the same petri plate cultures.

[e]Means followed by the same letter are not significantly different (P = 0.05) according to the Newman-Kuel procedure.

Table 2. Production of pseudothecia by Pyrenophora trichostoma in paired cultures with four growth inhibiting microorganisms on wheat straw incubated for 36 days at 16 C under constant light.

Treatment (P. trichostoma and indicated organism)	Number of pseudothecia[ab] per square cm
Bipolaris sorokiniana on sterile straw	11.34 x
P. trichostoma (ck) on non-sterile straw	14.34 x
Bacillus licheniformis on sterile straw	15.03 x
Alternaria tenuis on sterile straw	15.69 x
Stachybotrys chartarum on sterile straw	22.19 y
P. trichostoma (ck) on sterile straw	49.56 y

[a]Mean of four replicates.

[b]Numbers followed by the same letter are not significantly different (P = 0.05) according to Newman-Kuel procedure.

observed (Fig. 8). Consequently, a second experiment was performed in the manner of the one above except that in addition to pseudothecial counts, radial growth of PYOK-14 was measured toward the inhibitors and at right angles to the left and right. Means of these measurements, taken as representative growth of PYOK-14, were divided by the maximum growth (20 mm) of PYOK-14 without inhibitors to derive growth inhibition indices (Table 3). The paired growth indices (x's) and pseudothecial counts (y's) were used in a regression analysis to determine the relation of growth and pseudothecia production (Fig. 9). The results indicated that inhibition of growth of PYOK-14 by the microorganisms used in this study significantly reduced production of pseudothecia.

(f) Interaction of B. licheniformis with other microorganisms antagonistic to growth and reproduction of P. trichostoma. Bipolaris licheniformis significantly inhibited growth of A. tenuis, B. sorokiniana and S. chartarum (Table 4). The data indicated that growth of A. tenuis and B. sorokiniana were equally affected, and significantly more so than was S. chartarum.

The antimicrobial substance produced by B. licheniformis, as indicated by the ASI, differed significantly is its effect on the three fungi. The strongest interaction occurred between B. licheniformis and B. sorokiniana, the two most effective inhibitors of PYOK-14; and weakest interaction occurred between B. licheniformis and A. tenuis, the latter being the least inhibitory to growth of PYOK-14. These data suggest a possibility of mutually antagonistic relationships existing between B. licheniformis, B. sorokiniana and PYOK-14 (P. trichostoma).

(g) The effect of B. licheniformis on infection of wheat seedlings inoculated with P. trichostoma. Seedlings of wheat cultivar TAM W-101

Table 3. Growth and pseudothecial production of *Pyrenophora trichostoma* grown singly (cult. 1-4) and in dual cultures with antagonists *Alternaria tenuis* (cult. 5-8), *Stachybotrys chartarum* (cult. 9-12), *Bipolaris sorokiniana* (cult. 13-16), and *Bacillus licheniformis* (17-20).[a]

Culture Number	(x) Growth Ratio[b]	(y) Number of pseudothecia	Culture Number	(x) Growth Ratio[b]	(y) Number of Pseudothecia
1.	1.00	335	11.	0.50	102
2.	1.00	304	12.	0.58	4
3.	1.00	285	13.	0.28	9
4.	1.00	251	14.	0.50	34
5.	0.48	31	15.	0.35	85
6.	0.33	44	16.	0.45	17
7.	0.58	39	17.	0.20	4
8.	0.50	42	18.	0.33	3
9.	0.33	105	19.	0.28	2
10.	0.58	165	20.	0.33	3

[a] All cultures were grown on steam sterilized 20 mm segments of wheat straw placed side by side on moist filter paper in petri dishes.

[b] Growth ratios were calculated by dividing the mean of growth measurements toward the antagonists, and at right angles to the left and right of the inoculum, by the radial growth (20 mm) in singly grown cultures of *P. trichostoma*.

Table 4. Inhibitory effect of *Bacillus licheniformis* on three fungi antagonistic to growth of *Pyrenophora trichostoma*.[ab]

Treatment	Growth Inhibition Index[c]	Antimicrobial Substance Index[d]
B. licheniformis + *Alternaria tenuis*	0.52 x	0.16 x
B. licheniformis + *Stachybotrys chartarum*	0.78 y	0.21 y
B. licheniformis + *Bipolaris sorokiniana*	0.51 x	0.27 z

[a]Average of four replicates.

[b]Numbers followed by the same letters are not significantly different (P = 0.01) according to the Newman-Kuel procedure.

[c]Growth inhibition index (GII) = radial growth of fungal antagonist toward *B. licheniformis* ÷ radial growth in the opposite direction.

[d]Antimicrobial substance index (ASI) = width of inhibition zone ÷ radial growth of fungal antagonist opposit *B. licheniformis*.

Figure 8. Inhibition of growth and pseudothecial production of Pyrenophora trichostoma by 4-different microorganisms: P = Pyrenophora trichostoma, H = Bipolaris sorokiniana, S = Stachybotrys chartarum, A = Alternaria tenuis. NP = Non-sterile straw plus Pyrenophora trichostoma. Growth inhibition zones are especially evident in the PH and PS combinations. Note paucity of pseudothecia in all cultures except where P. trichostoma was cultured singly (P).

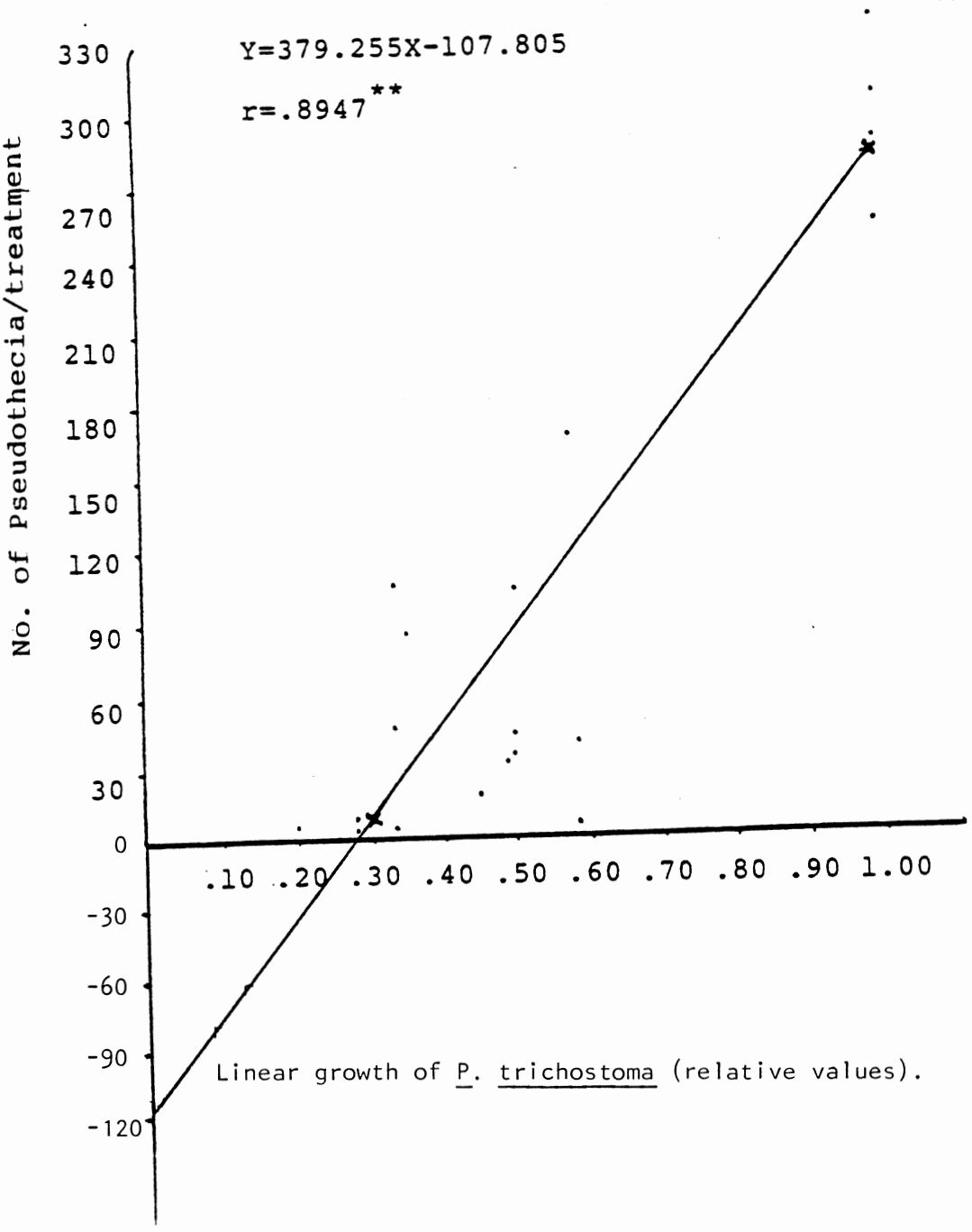

Figure 9. Linear regression between numbers of pseudothecia and linear growth of Pyrenophora trichostoma

inoculated with PYOK-14 developed numerous small chlorotic spots which rapidly expanded, coalesced, and became necrotic within 7 days. In many instances, the leaves became almost totally necrotic and died within 14 days. By contrast, plants inoculated and then sprayed immediately with 7 day old cultures of B. licheniformis in V-8 juice medium remained almost completely free of tan spot symptoms. Uninoculated check plants sprayed only with B. licheniformis and its V-8 juice also remained healthy. The test was repeated twice with similar results. The results (Fig. 10) indicated that B. licheniformis has potential as a biocontrol agent of tan spot and possibly other plant diseases.

(h) The effects of different concentrations of B. licheniformis culture filtrate on growth of P. trichostoma are shown in Tables 5 and 6. Growth of P. trichostoma was reduced on V-8 juice agar. A 1% concentration of the bacterial culture filtrate significantly reduced growth of the fungus. In liquid V-8 juice medium, a 0.125% dilution of the original filtrate caused an inhibition of growth. The ED_{50} (effective dilution to cause a 50% reduction in growth) was 9.2×10^{-3} as calculated by use of a regression equation (Fig. 11).

Culture filtrates of V-8 juice medium yeast extract medium, and Czapek dox broth medium showed different degrees of inhibition when tested against P. trichostoma.

Inhibition was greatest when Czapek dox broth was used as the culture medium and when the cultures were 9 days old. At this time, the filtrates had become dark gray in contrast to a light milky appearance of filtrates of 4 day old cultures.

Dark filtrates from cultures of B. licheniformis, grown in shake cultures (100 strokes/min) at room temperature for 7 days and having a

Table 5. Mycelial growth (linear) of Pyrenophora trichostoma grown in V-8 juice agar medium ammended with different concentrations of Bacillus licheniformis culture filtrate (Czapek dox broth).

Filtrate Concentration (%)	Radial Growth (mm)	Growth Reduction (%)
60.0	11.75	86.0
40.0	15.25	82.0
20.0	19.50	77.0
10.0	22.75	74.0
1.0	38.50	55.0
00 (check)	85.00	0.0

[a]Estimated number of cells in undiluted culture medium was 7.48 x 10'' per ml as determined by use of serial dilution plates.

[b]Average of four replicates.

Table 6. Mycelial growth (dry out) of Pyrenophora trichostoma grown in liquid V-8 juice medium ammended with different concentrations of Bacillus licheniformis culture filtrate (Czapek dox broth).

Filtrate Concentration[a] (%)	Mycelial Weight[b] (mg)	Growth Reduction (%)
1.0	45.7	60.0
0.5	93.5	18.0
0.25	104.4	9.0
0.125	113.6	0.4
0.0 (check)	114.1	0.0

[a]Estimated number of cells in undiluted culture medium was 7.48×10^{11} per ml as determined by use of serial dilution plates.

[b]Average of three replicates.

Figure 10. Control of Wheat tan spot caused by Pyrenophora trichostoma by Bacillus licheniformis.

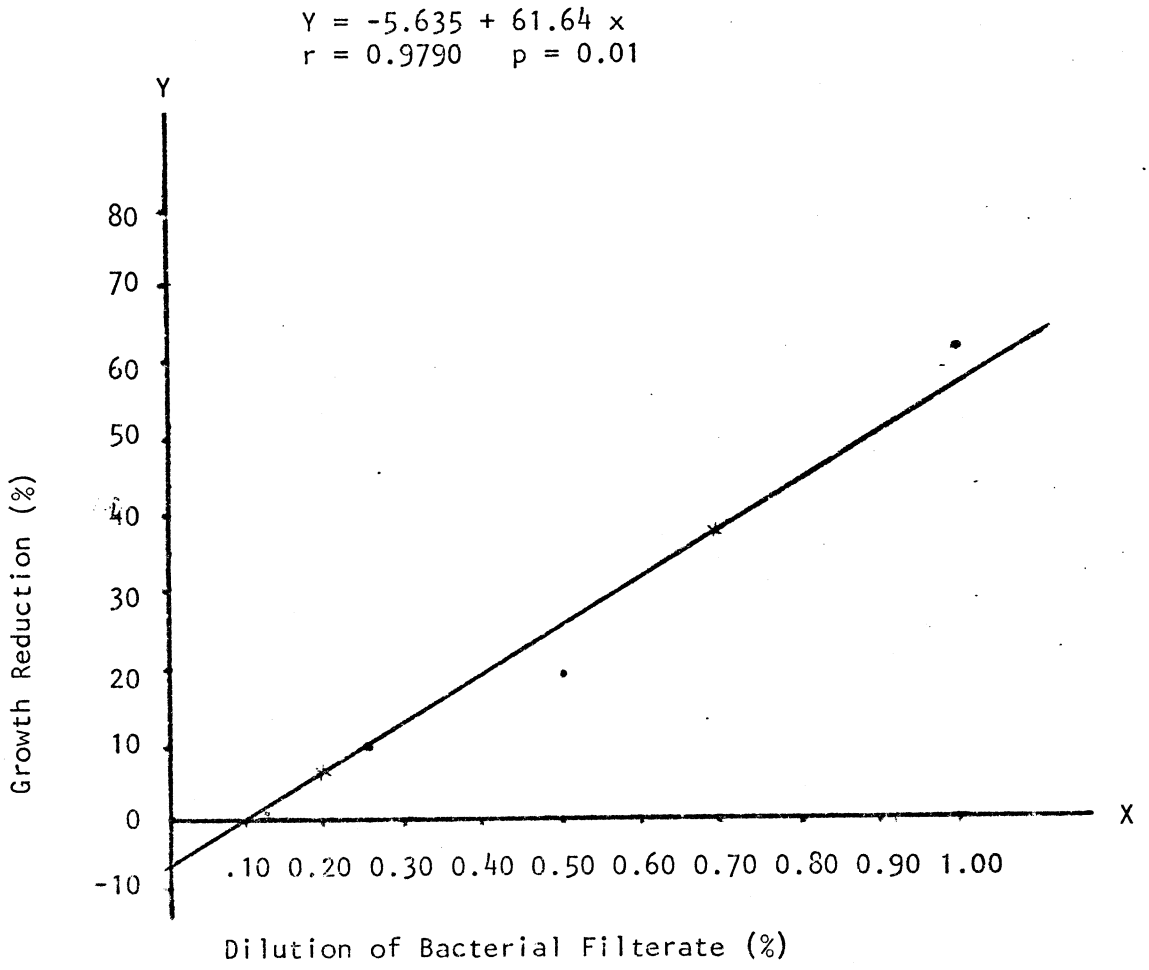

Figure 11. Linear regression between dilution of bacterial filterate (%) and growth reduction (%) of P. trichostoma.

viable cell count of 7.48×10^{11} per ml (determined by using serial dilution plates), showed more inhibitory activity against P. trichostoma than light colored ones.

Culture filtrate of B. licheniformis showed tolerance to heat. When the culture filtrate was added to V-8 agar and autoclaved at 121 C for 20 min the inhibitory effect on P. trichostoma was only slightly reduced.

The inhibitory property of B. licheniformis culture filtrate did not change after precipitation with 60% ammonium sulfate and dialysis against phosphate buffer.

A sephadex gel filtration column was packed and loaded twice. In the first trial, six fractions were collected and assayed for inhibition. In the first trial, inhibitory activity and mycelial lysis of P. trichostoma was detected in the sixth fraction. Void volume (Vo) collected for the column was 21.1 ml; while the elution volume (Ve) measured by detection of the inhibition and mycelial lysis zone was 44.1 ml. Total volume of the packed bed (vt) was 61.6 ml. By using these values in the formula

$$Kav = \frac{Ve - Vo}{Vt - Vo},$$

Kav was calculated to be 0.58. Reference to a selectivity curve for sephadex, G-type (Gel Filtration, Theory and Practrio; Pharmacia Fine Chemicals) indicated that the molecular weight of the active substance was about 3×10^4. In the second trial the column was repacked and 11 fractions collected. The collected void volume measured 22 ml. Inhibitory activity was observed in fractions 3, 4 and 5 with maximum activity in fraction four. The elution volume was 47 ml and the total packed

volume was 64.65. Using these values in the formula above, the Kav was calculated to be 0.58. Again referring to the selectivity curve for the gel (same reference as above) the molecular weight of the active substance was indicated to be about 3×10^4.

CHAPTER V

DISCUSSION

The work reported herein was a first attempt to identify naturally occurring microorganisms antagonistic to P. trichostoma, the casual agent of tan spot of wheat. P. trichostoma is generally considered as a foliar pathogen. However, in terms of time, P. trichostoma spends the major portion of its existence as a saprophyte in residue of its host plants. In Oklahoma, the foliar pathogen phase exists only from about mid-March, when ascospores are first released, until the wheat plants mature in mid-June. During the remaining months of the year, the fungus lives saprophytically in dead leaves and straw above and on the soil surface.

The primary goal of this study was to screen a broad spectrum of organisms for antagonistic activity against P. trichostoma. Consequently V-8 agar, was used. Although the medium supported representatives of bacteria, actinomycetes, and filamentuous fungi, it is recognized that it was not favorable for growth of all microflora in the soil, and that one to several antagonists of P. trichostoma may have gone undetected. Thus, the observed antagonists represented only those microorganisms efficiently capable of utilizing V-8 agar as a nutrient source.

B. licheniformis was the most potent antagonist against P. trichostoma among the organism most commonly isolated from soil on plant parts. B. licheniformis is placed in the family Bacillaceae in the 8th edition

of Bergy's Manual. Its important features are: the production of endospores, the production of antibiotics of the polypeptide class, and an energy releasing respiratory metabolism. Its spores occur in soil and may survive heat treatment (19). Ten forms of the antibiotic bacitracin are produced by this bacterium, among these bacitracin A is the most effective (16).

Among fungal antagonists isolated from wheat straw, Bipolaris sorokiniana, Stachybotrys chartarum, and Alternaria tenuis were observed most frequently.

B. sorokiniana is responsible for leaf spotting and blighting and for root rots of cereals and grasses. Although information concerning pathogenicity of B. sorokiniana is common in current or recent literature, reports regarding its production of toxins and antibiotics are limited. Pringle (42) reported that filtrates of 46 isolates of B. sorokiniana grown in liquid synthetic media produced necrosis in 27 lines of barley. He suggested that two types of toxins may be involved in the etiology of barley spot blotch. Yoder (59) discussed mechanisms and effects of toxins produced by different species of Helminthosporium. It was suggested that toxin produced by H. maydis acts specifically on mitochondria of sensitive cells, both in vivo and in vitro. Although my experiments did not include investigations of toxin production by B. sorokiniana, toxins secreted into the medium of paired cultures may possibly account for inhibited growth of P. trichostoma from a distance of 5 mm or more.

Strains of Stachybotrys produce compounds reported to be agents of stachbotryotoxicoses in animals and man (43). The toxins belong to the series of sequiterpenoid mycotoxins classified as 12,12-epoxy-Δ^{9}-

trichothecenes (43). Many members of Stachybotrys commonly found in soil are capable of utilizing celluose and damaging fabrics made of plant fibers (57). Also, clearing of cellulose agar medium by S. chartarum indicates that the fungus is a strong decomposer of cellulose (28).

Development of narrow zones of inhibition between colonies of P. trichostoma and S. chartarum in my experiments indicated the production of growth inhibiting substances which may have been produced by either or both organisms.

A. tenuis (=A. alternata) has been shown (50) to produce toxic substances belonging to three classes of compounds: tenazonic acid; dibenzo- -pyrones including alternariol, alternariol monoethyl ether, and altenuere; alteratoxins I and II which are toxic substances of unknown structure. A. tenuis produces the first and second types of toxins, but strains differ in the amount of toxin they produce. The third type of toxins are produced by some strains but not by others (50).

Growth inhibition zones did not develop between paired colonies of A. tenuis and P. trichostoma in my experiments. However, there was a slight reduction in growth of P. trichostoma which could have resulted from either direct competition for nutrients or from a mild reaction to toxin.

The methods used in my experiment precluded detection of all microorganisms in the soil samples that may have been antagonistic to growth of P. trichostoma. Firstly, the V-8 agar medium, while supportive to a wide spectrum of microbes, was probably not an optimum one for some antagonistic organisms. For example, two actinomycetes differing in color (one bluish-gray and one tan) were isolated which exhibited moderate to strong inhibition of growth relative to B. licheniformis. However, they

were not included in the present study because they were observed infrequently. Secondly, the strong inhibitory activity of B. licheniformis, when present as three or more colonies, often completely stopped growth of P. trichostoma thereby effectively masking possible antagonistic effects of other microorganisms. Thirdly, activity of B. licheniformis was not limited solely to P. trichostoma which resulted in reduced growth and possibly the inhibitory action of other antagonists of P. trichostoma. These factors contributed, at least in part, to selectivity of the inhibitory microorganisms.

The numbers of pseudothecia produced by P. trichostoma grown on non-sterile straw and on sterile straw in dual cultures with the antagonists was reduced significantly compared to the numbers produced by P. trichostoma cultured singly on sterile straw. By contrast, there was no significant difference in pseudothecia numbers produced by P. trichostoma on non-sterile straw and on sterile straw inoculated additionally with either B. licheniformis, B. sorokiniana, A. tenuis, at the 0.05 level of confidence. Pseudothecia produced in dual culture with S. chartarum was intermediate to the numbers in dual cultures with other antagonists and those produced in single culture on sterile straw. The apparent minor effect of S. chartarum on reduction of pseudothecia was attributed to its slow growth rate.

Reduced pseudothecial production and growth of P. trichostoma induced by the antagonists were linearly related. However, reduction in pseudothecial numbers probably was not the direct result of reduced growth. On the contrary, growth stress appeared to enhance pseudothecia development, viz.; temperature below the optimum for growth induced maturation of pseudothecia; on agar in petri dishes, single cultures

produced initials, or immature pseudothecia, most abundantly in aerial hyphae along the walls of the dishes; and incisions or UV radiation of mycelium appeared to induce pseudothecia to form. The stimulatory mechanisms of pseudothecial production under these conditions have not been elucidated, but they must include factors of the external (water potential, nutrient supply, etc.) and internal (enzyme systems) milieu. Also, biological entities such as bacteria (Ghazanfari, unpublished) have been observed to stimulate production of pseudothecia by P. trichostoma.

Notwithstanding, the capacity of these organisms to reduce pseudothecial numbers on straw in vitro, the extent of their effectiveness in nature may rely on several factors. Firstly, when present together one organism may suppress the activity of the others, or they may interact among themselves as either mutually enhancing or mutually mitigating agents of pseudothecia production. Secondly, stimulation of pseudothecia by unidentified microorganisms may nullify their effectiveness. Thirdly, environmental conditions may favor one organism more than another. Fourthly, in nature pseudothecia of P. trichostoma are truly erumpent and effectiveness of the antagonists may relate directly to the depth that their cells, or antibiotic substances they produce, penetrate the tissue of the straw. Also, if mutualistic antagonism exists between P. trichostoma and the other microorganisms, the earliest invader of the plant tissue would probably have a territorial advantage (5). Since P. trichostoma is a proficient pathogen of living tissue, it may be assumed that it would occupy the straw tissue prior to colonization by B. sorokiniana, S. chartarum, A. tenuis and B. licheniformis.

Filtrates from cultures of B. licheniformis reduced growth and

caused mycelial cell lysis of P. trichostoma. Heat tolerance, dializability precipitation in amoniumun sulfate and a high molecular weight indicated that the active principle in the filtrate may be a low molecular weight protein, a polysaccharide or polynucleotide, or a combination of these.

Several reports of cell lysis of fungi by bacteria are reported in the literature. A Bacillus isolated from stem and leaf rust pustules destroyed pycenia, aecia, and uredia by lytic action (30). Three different bacteria have been shown capable of lysing germtubes of rust urediospores (36), and filtrates of all three lysed germ tubes. However, the filtrate from only one species, B. pumilus, was able to cause lysing after autoclaving. This indicated a substance other than an enzyme was involved.

B. subtilis is antagonistic to the apple canker pathogen, Nectria galligena, in leaf scar tissue (51). Isolates of B. subtilis from leaf scar tissue produced two antifungal and eight antibacterial antibiotics, but only the former were sufficiently stable to create inhibition zones on agar. The antifungal components inhibited spore germinations of several saprophytic fungi in vitro. If germination occurred in the presence of the antibiotics, as in the case with N. galligena, the emerging hyphae swelled and burst (52).

Among the microorganisms identified as inhibitors to growth and reproduction of P. trichostoma in this study, B. licheniformis appears to offer the greatest potential as an agent for biological control of tan spot and possibly other diseases. It almost completely inhibited infection of wheat seedlings by P. trichostoma when applied as a cell suspension in liquid culture medium. But in addition to strong antag-

onistic properties on leaves and in agar culture, a propensity to form heat tolerant spores, its ability to produce antimicrobial substance and an ability to utilize inexpensive substrates indicate that efforts to exploit B. licheniformis as a bio-control agent would have a high probability of success.

Summary

Interactions of Pyrenophora trichostoma, the cause of tan spot of wheat with four microorganisms isolated from wheat field soils and wheat straw was studied. The following is a summary of results obtained from this study.

1. Bacillus licheniformis isolated from wheat field soil and from foliage of wheat plants was strongly inhibitory to growth of P. trichostoma on V-8 agar and to growth and sexual reproduction on sterilized wheat straw. The nearly complete inhibition of growth of P. trichostoma from a distance of 20 mm indicated from B. licheniformis released a highly active antifungal substance that could be of substantial benefit to wheat production.

2. Bipolaris sorokiniana, Stachybotrys chartarum, and Alternaria tenuis, isolated from wheat straw also inhibited growth of P. trichostoma on V-8 agar and sterilized straw. B. licheniformis and B. sorokiniana were significantly superior to S. chartarum and A. tenuis as growth inhibitors.

3. B. licheniformis stopped radial growth of P. trichostoma in paired cultures from a distance of 12-20 mm with never an intermingling of colonies, B. sorokiniana and S. chartarum stopped growth of P. trichostoma at a lesser distance without mycelial contact. By contrast,

A. tenuis, hyphae intermingled with that P. trichostoma indicating that reduced growth of P. trichostoma probably derived from direct competition for nutrients rather than from the production of antibiotics or toxic substances.

4. The average numbers of pseudothecia produced by P. trichostoma on sterile wheat straw, non-sterile field straw, and on sterile straw inoculated additionally with each growth inhibition microorganism fitted into three nearly discreet groups at the 0.05 confidence level; the groups in descending order of pseudothecia production were: (a) sterile straw, (b) paired culture with S. chartarum, and (c) paired culture with the three remaining microorganisms.

5. Paired culture growth indices and pseudothecial counts were used in a regression analysis to determine the relation of growth and pseudothecial production. The results indicated that inhibition of growth of P. trichostoma by the microorganisms significantly reduced production of pseudothecia.

6. B. licheniformis significantly inhibited growth of A. tenuis, B. sorokiniana and S. chartarum. The data indicated that growth of A. tenuis and B. sorokiniana were equally affected, and significantly more so than was S. chartarum.

7. Seedlings of wheat cultivar TAM W-101, inoculated with P. trichostoma, developed numerous small chlorotic spots which rapidly expanded, coalesced and became necrotic. By contrast, plants inoculated and then sprayed immediately with a 7 day old culture B. licheniformis in V-8 juice medium remained almost completely free of tan spot symptoms.

8. Diluted culture filtrate of B. licheniformis grown in Czapek dox broth reduced growth of P. trichostoma at 0.125 percent strength,

the lowest concentration tested. An ED_{50} (dilution at which growth was reduced 50%) of 9.2×10^2 was calculated for the partially purified growth inhibiting substance. The inhibitory substance was heat stable, precipated in a saturated amonium sulfate solution, and had a molecular weight of about 3×10^4 as indicated by use of a sephadex gel column.

LITERATURE CITED

1. Andrews, E., and W. Klomparens. 1952. Yellow spot of winter wheat in Michigan in 1951. Plant Dis. Reptr. 35:10-11.

2. Baker, K.F., and Cook, R.J. 1974. Biological Control of Plant Pathogens. W.H. Freeman, San Francisco. 433 pp.

3. Barr, Margaret E. 1977. A classification of Loculoascomycetes. Mycologia. 71:935-937.

4. Basham, J.T. 1978. Early sugar maple stem discoloration and microorganism invasion in simulated wounds of felling and fire scars. Phytopathology 68:1643-1699.

5. Bruel, G.W., and P. Lai. 1966. Prior-colonization as a factor in the saprophytic survival of several fungi in wheat straw. Phytopathology 56:766-768.

6. Bisset, K.A. and Robert Bartlett. 1978. The isolation and characters of L-forms and reversions of B. licheniformis endoparasiticus associated with the erythrogates of clinically normal persons. J. Med. Microbiol. 11:335-349.

7. Blakeman, J.P. and I.D.S. Brodie. 1976. Inhibition of pathogens by epiphytic bacteria on aerial plant surfaces. Pages 529-557 in: C.H. Dickinson and T.F. Preece, eds. Microbiology of aerial plant surfaces. Academic Press, New York. 669 pp.

8. Broadent, D. 1968. Antibiotics produced by fungi. Pest articles and news summaries Section B. 14:120-141.

9. Cappellini, R.A., A.W. Stretch and J.M. Maiella. 1972. Fungi associated with blueberries held at various storage times and temperatures. Phytopathology 62:68-69.

10. Conners, I.L. 1939. Yellow leaf blotch. 19th Annual Rep. Can Plant Dis Surv., Pages 12-14.

11. Conners, I.L. 1967. An annotated index of plant diseases in Canada. Can. Dep. Agr. Publ. 1251. 381 pp.

12. Dikison, C.H. 1976. Fungi on the aerial surfaces of higher plants. Pages 293-324 in: C.H. Dikinson and T.F. Preece, eds. Microbiology of aerial plant surfaces. Academic Press, New York. 669 pp.

13. Dikinson, C.H. and D. Bottomley. 1978. Germination and growth of Alternaria and Cladosporium in relation to their activity in the phylloplane. Trans. Br. Mycol. Soc. 74:309-319.

14. Dikinson, C.H. and B. Wallace. 1976. Effects of late application of foliar fungicides on activity of microorganisms on winter wheat flag leaves. Trans. Br. Mycol. Soc. 66:103-112.

15. Dugg, A.D.S. 1954. A new disease of wheat in Kenya caused by a species of Pyrenophora. East Afr. Agric. J. 19:225-228.

16. Flickinger, M.C. and D. Perlman. 1979. Application of oxygen enriched aeration in the production of bacitracin by Bacillus licheniformis. Antimicrob. Agents and Chemother. 15:282-293.

17. Fokkema, N.J. 1976. Antagonism between fungal saprophytes and pathogens on aerial plant surfaces. Pages 487-506 in: C.H. Dickinson and T.F. Preece, eds. Microbiology of aerial plant surfaces. Academic Press, New York. 669 pp.

18. Fries, N. 1973. Effects of volatile organic compounds on the growth and development of fungi. Trans. Br. Mycol. Soc. 60:1-21.

19. Gibsen, T. and Ruth E. Gordon. 1974. Endospore forming rods and cocci. Pages 529-535 in: Bergy's Manual of Determinative Bacteriology. 8th ed. Baltimore, 1246 pp.

20. Gottlief, D. and P.O. Shaw. 1970. Mechanism of action of anti-fungal antibiotics. Ann. Rev. Phytopathol. 8:371-402.

21. Hosford, R.M. Jr. 1971. A form of Pyrenophora trichostoma pathogenic to wheat and other grasses. Phytopathology 61:28-32.

22. Hosford, Robert M., Jr. 1972. Propagules of Pyrenophora trichostoma. Phytopathology 62:627-629.

23. Hosford, R.M., Jr. 1976. Fungal leaf spot diseases of wheat in North Dakota. N. Dakota Agric. Exp. Stn. Bull. 500.12 pp.

24. Hosford, R.M., Jr. and R.H. Busch. 1974. Losses in wheat caused by Pyrenophora trichostoma and Leptosphaerva avenaria f. sp. tritici. Phytopathology 64:184-187.

25. Hutchinson, S.A. 1973. Biological activities of volatile fungal metabolites. Ann. Rev. Phytopathol. 11:223-246.

26. International maze and wheat improvement center (CIMMYT) Annu. Rep. 1968-1969. Morocco. pp. 96-98.

27. Joly, Patrick. 1967. Key for determination of the most common species of the genus Alternaria (nees) wiltsh. Emend. Joly. Plant Dis. Rep. 51:296-298.

28. Jong, S.C. and E.E. Davis. 1976. Contribution to the knowledge of Stachybotrys and memnoniella in culture. Mycotaxon. 3:407-485.

29. Kenneth, R. 1958. Contribution to the Knowledge of the Helminthosporicom flora on gramineae in Israel. Res. Counc. Israel Bull. 6, D, 4. pp. 191-210.

30. Levine, M.N., R.H. Bamburg and R.E. Atkinson. 1936. Microorganisms antibiotic or pathogenic to cereal rusts. Phytopathology 26:99-100.

31. Lindewfelser, L.A. and A. Giegler. 1969. Production of antibiotics by Alternaria species. Developments in Industrial Microbiology. 10:271-278.

32. Luzzardi, G.C. and W.C. da Luz. 1976. Occorrencia de' Helminthosporium tritici-repentis Died. em semestes, no Rio Grand do Sul. VIII Reoniao Anual Conjunta de Pesquisa de trigo. Ponta Grossa, Brazil. 2 pp.

33. Mehta, Y.R. 1978. Doencas do trigoe seu controle. Summa Phytopathol. Editora Agronomica Ceres Ltda. Brazil. 190 pp.

34. Mishra, R.R. and R.P. Tewari. 1976. Studies on biological control of Puccinia graminis tritici. Pages 559-567 in: C.H. Dikinson and T.F. Preece eds. Microbiology of Aerial plant surfaces. Academic Press, New York. 669 pp.

35. Misra, A.P. and R.A. Singh. 1972. Pathogenic differences amongst three isolates of Helminthosporium tritici repentis and the preference of wheat varieties against them. Indian Phytopathol. 25:350-353.

36. Morgan, F.L. 1963. Infection inhibition and germ-tube lysis of three cereal rusts by Bacillus pamilus Phytopathology 53:1346-1348.

37. Nisidado, Y. 1929. Preliminary notes on yellow spot disease of wheat caused by Helminthosporium tritici vulgans Nisikado. Berline Ohara Inst. Landw. Forsch. 4:103-104.

38. Odvody, G.N. and M.G. Boosalis. 1978. The development and maturation of pseudopenthecia of Pyrenophora trichostoma on wheat straw in Nebraska. Phytopathol. News. 12:8.

39. Parisinos, J. 1956. Wheat and barley production in Cyprus (Part II). Countryman, Nicosia. pp. 12-13.

40. Porter, C.L. 1924. Concerning the characters of certain fungi as exhibited by their growth in the presence of other fungi. Bot. 11:168-188.

41. Platt, H.W., R.A.A. Morrall and H.E. Gruen. 1977. The effects of substrate, temperature, and photperiod on condition of Pyrenophora tritici-repentis. Can. J. Bot. 55:254-259.

42. Pringle, Ross B. 1980. Role of toxins in etiology of spot blotch disease of barley. Can. Plant Dis. Surv. 59(4):74-79.

43. Rodericks, J.F. and R.M. Eppley. 1974. Stachybotrys and stachybotryotoxicosis. Pages 181-197 in: I.F.H. Purchase, ed. Mycotoxins. Elsevier Scint. Publ. Co., New York.

44. Sharp, E.L., B.K. Sally, and F.H. MacNeal. 1976. Effect of Pyrenophora wheat leaf blight on the thousand kernel weight of 30 spring wheat cultivars. Plant Dis. Rep. 60:135-138.

45. Shaw, D.E. and P.G. Valder. 1953. A study of the microflora of wheat grains in New South Wales. Proc. Linn. Soc. N.S.W. 77:307-322.

46. Shoemaker, R.A. 1959. Nomenclature of Drechslera and Bipolaris, grass parasites segregated from Helminthosporium. Can. J. Bot. 37:879-887.

47. Skidmore, A.M. 1976. Interactions in relation to biological control of plant pathogens. Pages 507-528 in: C.H. Dikinson and T.F. Preece, eds. Microbiology of Aerial Surfaces. Academic Press, New York. 669 pp.

48. Skidmore, A.M. and C.H. Dickinson. 1976. Colony interactions and hyphal interference between Septoria nodorum and phylloplane fungi. Trans. Br. Mycol. Soc. 66:57-64.

49. Sprague, R. 1950. Diseases of Cereals and grasses in North America. Ronald Press, New York, N.Y. 538 pp.

50. Stinson, E.E., D.D. Bills, S.F. Osman, J. Siciliano, M.J. Ceponis and E.G. Heisler. 1980. Mycotoxin production by Alternaria species grown on apples, tomatoes, and blueberries. J. Agric. Food Chem. 28:960-963.

51. Swinburne, T.R. 1973. Microflora of apple leaf scars in relation to infection by Nectria galligena. Trans. Br. Mycol. Soc. 60:389-403.

52. Swinburne, T.R., J. Barr and A.E. Brown. 1975. Production of antibiotics by Bacillus subtilis and their effect on fungal colonists of apple leaf scars. Trans. Br. Mycol. Soc. 65:211-217.

53. Tekauz, A. 1976. Distribution, severity, and relative importance of leaf spot diseases of wheat in western Canada in 1974. Can. Plant Dis. Surv. 56:36-40.

54. Wallace, G.B. and M.M. Wallace. 1954. Tanganyika fungus list: recent records NO. XVIII Tanganyika . Dep. Agr. Mycol. Circ. No. 36:7 pp.

55. Watkins, J.E., G.N. Odvody, M.G. Boosalis, and J.E. Partridge. 1978. An epidemic of tan spot of wheat in Nebraska. Plant Dis. Rep. 62:132-134.

56. Wehmeyer, L.E. 1961. A world Monograph of the genus Pleospora and its segregates. The University of Michigan Press. 451 pp.

57. Wiese, M.V. 1977. Compendium of wheat diseases. Am. Phytopathol. Soc. St. Paul, MN. 106 pp.

58. Yoder, O.C. 1980. Toxins in pathogenesis. Ann. Rev. Phytopathol. 18:103-29.

VITA

Jamshid Ghazanfari-Hesamabadi

Candidate for the Degree of

Doctor of Philosophy

Thesis: INTERACTIONS OF THE TAN SPOT FUNGUS, PYRENOPHORA TRICHOSTOMA, AND SELECTED MICROORGANISMS

Major Field: Plant Pathology

Biographical:

Personal Data: Born in Shiraz, Iran, May 24, 1943, the son of Mr. and Mrs. Heidar Ghazanfari-Hesamabadi; married to Mahin Shayegan, father of three sons, Kambiz, Hooman and Babak.

Education: Attended high school at Shahpoor school, Shiraz, graduated in 1962; received Bachelor of Science degree in Agriculture from Pahlavi University in 1967; received Master of Science degree from Pahlavi University in 1972; completed requirements for the Doctor of Philosophy degree at Oklahoma State University in July, 1983.

Professional Experience: Extension Specialist in Ministry of Agriculture for 2 years; military service, 1967-1969; plant protection specialist, Department of extension, Ministry of Agriculture, Iranian Government, 1969-1972; Instructor at Rezaieh (Uromieh) University, Uromieh, Iran, 1972-1978; taught two courses in Elementary and advanced plant pathology, Graduate student, Oklahoma State University, 1978-1983.